Biochemical Pharmacology, Vol. 18, pp 2019–2027. Pergamon Press. 1969. Printed in Great Britain

COMPARISON OF THE BIOCHEMICAL ALTERATIONS ELICITED IN LIVERS FROM RATS TREATED WITH CARBON TETRACHLORIDE, CHLOROFORM, 1, 1, 2-TRICHLOROETHANE AND 1,1,1-TRICHLOROETHANE*

CURTIS D. KLAASSEN† and GABRIEL L. PLAA‡

Oakdale Toxicology Center, Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, Iowa 52240, U.S.A.

(*Received* 12 *November* 1968; *accepted* 21 *February* 1969)

Abstract—Chlorinated hydrocarbons differ in their capacity to produce liver damage. The comparative effects of carbon tetrachloride (CCl_4), chloroform, 1,1,1-trichloroethane and 1,1,2-trichloroethane were studied in rats. CCl_4 produced the highest increase in hepatic triglycerides and also produced elevated levels at the lowest dose (0·03 ml/kg). Chloroform was intermediate in producing elevated liver triglyceride levels; with 1,1,2-trichloroethane, enhanced triglyceride levels were demonstrated only at near-lethal dosages. No enhanced hepatic triglyceride level was demonstrated with 1,1,1-trichloroethane. Significant dose-related decreases in glucose 6-phosphatase activity were demonstrated with doses of 0·3 ml/kg of CCl_4 or greater. With the other chlorinated hydrocarbons, no decrease in hepatic glucose 6-phosphatase activity was detected. When the hydrocarbons were added directly to liver homogenates, only those incubations containing CCl_4 exhibited increased lipid peroxidation (enhanced thiobarbituric acid reactants). *In vivo*, evidence of lipid peroxidation (diene conjugates) in the liver was obtained 15 min after CCl_4 treatment; this effect reached its peak at 30 min. Enhanced diene conjugates were detected with doses of 0·3 ml/kg of CCl_4 or greater. However, with the other three hydrocarbons, no increase in diene conjugates was detected. Thus, this study shows that while the temporal relationships of CCl_4-induced hepatotoxicity are compatible with the lipid peroxidation hypothesis, the dosage relationship is weaker. Also the lack of qualitative similarity in the results obtained with chloroform is disturbing.

A LARGE number of studies have been performed to determine the mechanism of carbon tetrachloride hepatotoxicity. The hypotheses concerning the mechanism of the fatty changes seen after carbon tetrachloride have been recently reviewed by Recknagel.[1] A current hypothesis is that the primary event in the hepatotoxic response is the homolytic cleavage of carbon tetrachloride to trichloromethyl free-radical and monatomic chlorine. This cleavage presumably takes place in or near the endoplasmic reticulum of the hepatic parenchymal cell, and the free-radicals are believed to attack the lipoidal elements of the endoplasmic reticulum, thus initiating the autocatalytic, peroxidative breakdown of these membranes. The liver cells are then unable to secrete triglycerides into the plasma, and the fatty liver develops.

* This work was supported by funds from USPHS Research Grant GM-12675.

† USPHS Predoctoral Fellow (5-Fl-GM-30,996). Present address: Department of Pharmacology, University of Kansas Medical School, Kansas City, Kansas.

‡ Present address: Department of Pharmacology, Faculty of Medicine, University of Montreal, Montreal, Canada.

Chlorinated hydrocarbons other than carbon tetrachloride also produce liver dysfunction. Klaassen and Plaa[2, 3] reported that in both mice and dogs carbon tetrachloride caused severe hepatic dysfunction, while moderate to severe changes were seen with chloroform; moderate alterations were seen with 1,1,2-trichloroethane, and only mild dysfunction was observed with trichloroethylene, tetrachloroethylene, dichloromethane and 1,1,1-trichloroethane, when administered at near-lethal doses.

The purpose of the present investigation was to study the temporal and dosage relationship in regard to the biochemical alterations seen after carbon tetrachloride and also to determine if similar changes could be exhibited after chloroform, 1,1,2-trichloroethane and 1,1,1-trichloroethane.

METHODS

Male, Sprague–Dawley rats (300–400 g) were used throughout. Analytical grades of the following hydrocarbons were employed: carbon tetrachloride; chloroform; 1,1,2-trichloroethane; and 1,1,1-trichloroethane. All agents were administered intraperitoneally and were made up in corn oil solutions to deliver the proper dosage in a final volume of 1 ml/100 g of body weight.

Lethality. For the 24 hr LD_{50} determinations, four or five groups of rats, five to ten per group, were injected with a graded series of doses of the hydrocarbons and the number of deaths was recorded at the end of 24 hr. The median lethal dose (LD_{50}) was then calculated for each hydrocarbon by the method of Litchfield and Wilcoxon.[4]

Liver triglycerides. Hepatic triglycerides were measured by a modified method of Van Handel and Zilversmit.[5]

Glucose 6-phosphatase. Activity of this enzyme was determined in rat liver homogenized in 0·1 M maleic acid (pH 6·25). Final conditions for the enzyme assay were as follows: 0·5 ml of 0·2 M disodium glucose 6-phosphate, pH 6·25; 1·5 ml of 0·1 M maleic acid buffer, pH 6·25; 0·5 ml liver homogenate, 20 mg/ml; 40 min of incubation at 37°. The reaction was terminated with the addition of 10 ml of 10% trichloroacetic acid. Inorganic phosphorus was determined in 2-ml aliquots of the supernatant by the method of Fiske and Subbarow,[6] using reagents obtained from the Sigma Chemical Company.

Lipid peroxidation in vitro. The peroxidation of rat liver homogenates after addition of various amounts of chlorinated hydrocarbons was determined by using the thiobarbituric acid (TBA) method of Comparti *et al.*[7, 8]

Final conditions for the enzyme assay were as follows: 2 ml of 0·15 M potassium phosphate buffer, pH 7·4; 1 ml of 0·15 M potassium chloride; 1 ml of 30% liver homogenate in ice cold 0·15 M KCl; varying volumes of the hydrocarbons; the flasks stoppered, incubated, and shaken for 45 min at 37°. The reaction was stopped by the addition of 4 ml trichloroacetic acid. After centrifugation, the amount of peroxide formed was estimated in 2·0 ml of supernatant by adding 2·0 ml of 0·67% TBA and boiling for 10 min. Optical densities were measured at 543 mμ.

Lipid peroxidation in vivo. The quantitative estimation of the lipid peroxidation *in vivo* was determined by measuring diene conjugate ultraviolet absorption of lipid extracts of the microsomal fraction of rat livers, as described by Reynolds (personal communication). Eight g of liver was homogenized with a Potter–Elvehjem homogenizer in 16 ml of ice-cold 0·3 M sucrose which was 3 mM with respect to EDTA. The homogenate was centrifuged at 9000 *g* for 20 min and 13 ml of the supernatant was

then centrifuged at 94,000 *g* for 60 min. The supernatant was decanted and the microsomal fraction was resuspended in 2 ml of the sucrose–EDTA solution. An aliquot of the microsomal suspension (0·5 ml) was added to 9·5 ml of a chloroform–methanol (2:1) mixture. After centrifugation, the supernatant was decanted into a graduated centrifuge tube and brought to a volume of 10 ml by the addition of chloroform–methanol mixture. Distilled water (2·0 ml) was added and the two phases were thoroughly mixed and then centrifuged. The upper phase was aspirated off and discarded. The interface was washed twice with 0·5 ml of fresh upper phase. Stock upper and lower phases were made each day by adding 25 ml water to 95 ml of the chloroform–methanol mixture and shaking. The upper phase was used for washing the interface, and the lower phase was used as a blank for spectrophotometry. Methanol (200 μl) was added to each tube and the absorbance of an aliquot was read at 243 mμ in a Gilford spectrophotometer.

Statistics. Means were compared by an analysis of variance. When a significant difference ($P < 0{\cdot}05$) was found, the means were then compared by the Student *t*-test.[9]

RESULTS

Lethality. The 24 hr LD_{50} values and their per cent confidence limits were as follows: 1,1,2-trichloroethane, 0·65(0·60 to 0·70) ml/kg; chloroform, 1·3 (1·1 to 1·4) ml/kg; carbon tetrachloride, 1·9 (1·7 to 2·1) ml/kg; and 1,1,1-trichloroethane, 3·8 (3·3 to 4·2) ml/kg. Similar LD_{50} values have been previously reported for these hydrocarbons in mice and dogs.[2, 3] These LD_{50} values were used in the later studies to calculate the 0·75 × LD_{50} doses used in the time-response studies: carbon tetrachloride, 1·4 ml/kg; chloroform, 0·94 ml/kg; 1,1,2-trichloroethane, 0·49 ml/kg; and 1,1,1-trichloroethane, 2·8 ml/kg.

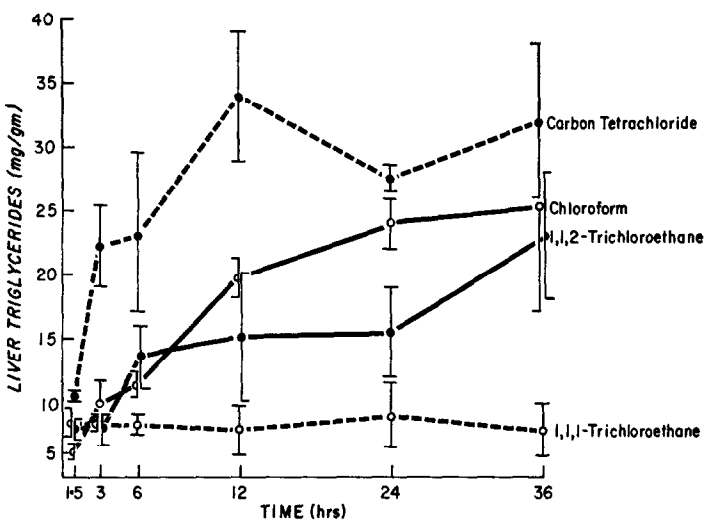

FIG. 1. Temporal accumulation of hepatic triglycerides (per g wet wt. of liver) after a high dose of each hydrocarbon (0·75 × LD_{50}). Each point represents the mean ± S.E. of three rats.

Liver triglycerides. Figure 1 demonstrates the 36 hr time response for the accumulation of hepatic triglycerides after treatment with the four hydrocarbons (0.75 × LD_{50}). The highest hepatic triglyceride levels were demonstrated after carbon tetrachloride treatment; enhanced levels were demonstrated 1·5 hr after its administration, which was the earliest time tested. The triglyceride level increased until the 12 hr interval and remained quite constant thereafter. Chloroform and 1,1,2-trichloroethane produced intermediate elevations in hepatic triglycerides and tended to increase throughout the 36-hr interval. With 1, 1, 1-trichloroethane, no increase in hepatic triglycerides was demonstrated at any time interval.

Figure 2 depicts the dose-response relatinship for the accumulation of triglycerides 24 hr after the administration of the chlorinated hydrocarbons. Carbon tetrachloride produced the highest hepatic levels of triglycerides and also produced

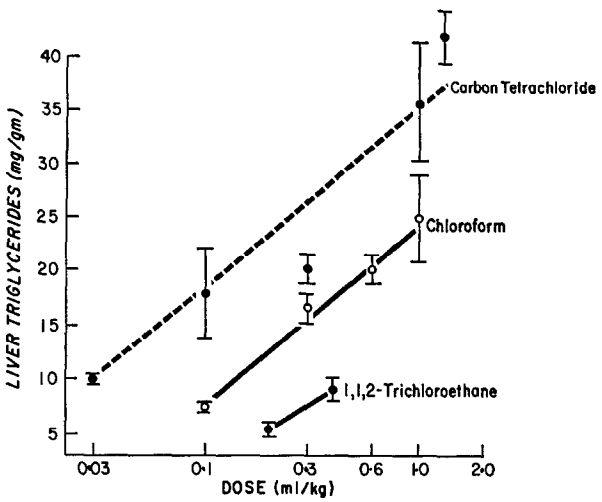

FIG. 2. Dose–response relationship for the accumulation of hepatic triglycerides (per g wet wt. of liver) 24 hr after administration of the hydrocarbon. Mean ± S.E. of three rats. Five doses were used for carbon tetrachloride, four for chloroform, and two for 1,1,2-trichloroethane.

elevated levels at the lowest dose. Chloroform was intermediate in producing enhanced liver triglyceride levels. With 1,1,2-trichloroethane enhanced triglyceride levels were demonstrated only at near-lethal doses.

Glucose 6-phosphatase. Figure 3 depicts the glucose 6-phosphatase activity of the liver of rats at various times after treatment with carbon etrachloride (0·75 × LD_{50}). Three hr after treatment, the enzyme activity was still normal, but by 6 hr it was depressed to below 50 per cent of control activity. The activity remained low for the rest of the time intervals tested. With the other chlorinated hydrocarbons, no decrease in glucose 6-phosphatase was exhibited at any of the time intervals. These values ranged from 9·9 ± 0·7 to 12·7 ± 1·2, 9·1 ± 2·5 to 14·8 ± 1·9, and 10·6 ± 1·1 to 13·8 ± 1·5 mg Pi/g, for chloroform, 1,1,2-trichloroethane and 1,1,1-trichloroethane respectively.

Figure 4 demonstrates the glucose 6-phosphatase activity 12 hr after varying doses of CCl_4. There was a significant decrease in activity from control levels in the rats treated with 1·0 and 0·3 ml/kg, but not with 0·1 and 0·03 ml/kg.

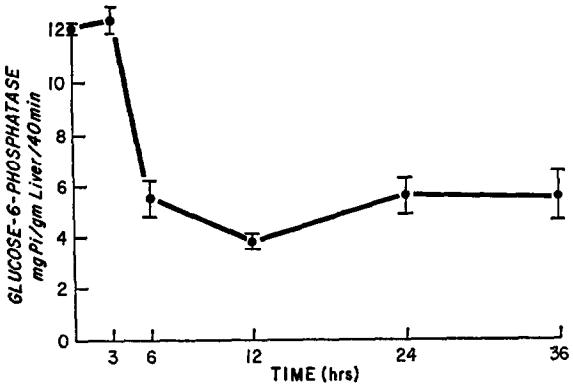

FIG. 3. Time–response relationship for glucose 6-phosphatase activity (per g wet wt. of liver) after a dose of CCl_4 (0·75 × LD_{50}). Mean ± S.E. of three rats.

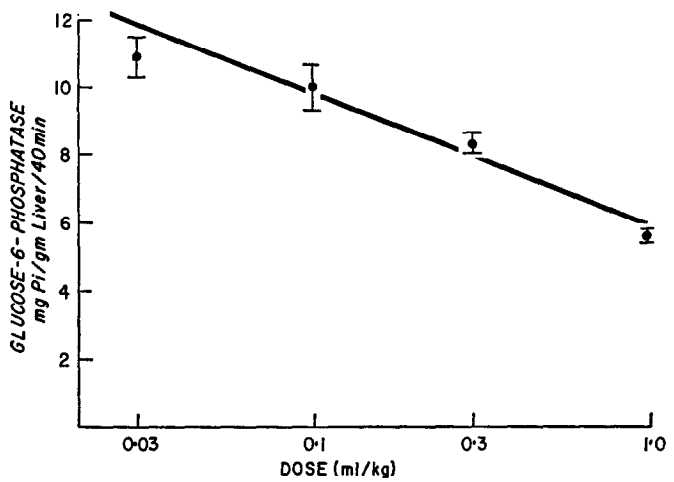

FIG. 4. Dose–response relationship for glucose 6-phosphatase activity (per g wet wt. of liver) 12 hr after CCl_4. Mean ± S.E. of three rats.

TABLE 1. EFFECT OF VARIOUS CONCENTRATIONS OF THE CHLORINATED HYDROCARBONS ON LIPOPEROXIDATION *IN VITRO*

Amt.* (μl)	Carbon tetrachloride	Chloroform	1,1,2-Trichloroethane	1,1,1-Trichloroethane
1	0·210 ± 0·024†‡	0·148 ± 0·024	0·151 ± 0·023	0·132 ± 0·021
2	0·236 ± 0·020‡	0·160 ± 0·024	0·111 ± 0·024	0·131 ± 0·019
5	0·268 ± 0·041‡	0·156 ± 0·017	0·095 ± 0·009	0·145 ± 0·028
10	0·290 ± 0·051‡	0·128 ± 0·16	0·072 ± 0·005§	0·177 ± 0·031‡
25	0·239 ± 0·072‡	0·093 ± 0·005	0·088 ± 0·006§	0·107 ± 0·013
50	0·111 ± 0·009	0·070 ± 0·005§	0·071 ± 0·008§	0·078 ± 0·007§
100	0·111 ± 0·011	0·079 ± 0·009§	0·080 ± 0·007§	0·068 ± 0·012§
200	0·112 ± 0·011	0·068 ± 0·009§	0·064 ± 0·008§	0·059 ± 0·010§

* Volume of hydrocarbon added to the incubation medium.

† Mean ± S.E. of the optical density of the TBA reactants of six treated rats. The mean ± S.E. of 24 control rat livers was 0·123 ± 0·008.

‡ Significantly greater ($P < 0·05$) concentration of TBA reactants than in control rat liver.

§ Significantly lower ($P < 0·05$) concentration of TBA reactants than in control rat liver.

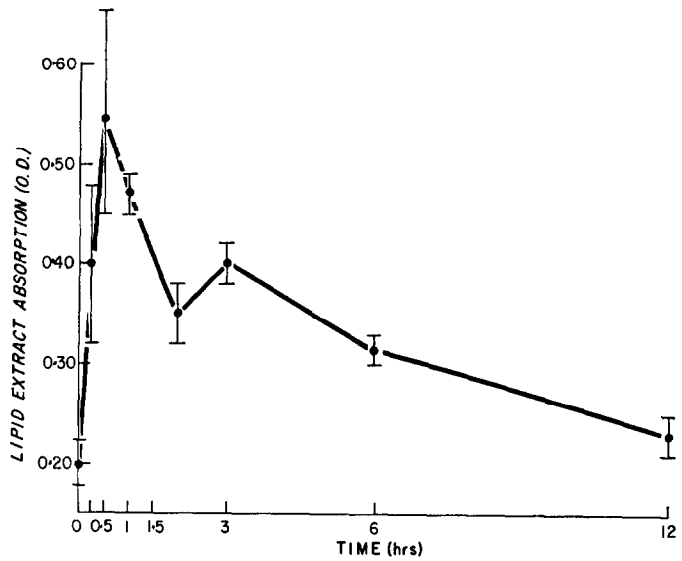

FIG. 5. Time–response relationship for appearance of diene conjugates in microsomal lipids after administration of CCl_4 (0·75 × LD_{50}). Mean ± S.E. of three rats.

Lipid peroxidation in vitro. Table 1 demonstrates the ability of the four chlorinated hydrocarbons to enhance peroxidation *in vitro*, as measured by the thiobarbituric acid method. Increased TBA reactants were detected after 1, 2, 5, 10 and 25 μl carbon tetrachloride, but not with 50, 100 and 200 μl. With chloroform and 1,1,2-trichloroethane, no enhanced lipid peroxidation was detected with any of the doses used. However, decreased lipid peroxidation was observed with the higher doses of these two hydrocarbons. With 1,1,1-trichloroethane, enhanced lipid peroxidation was detected with one dose of the hydrocarbon (10μl) and decreased with the higher doses (50–200 μl).

Lipid peroxidation in vivo. Figure 5 depicts the amount of lipid peroxidation detected at various time intervals in livers from rats treated with carbon tetrachloride (0·75 × LD_{50}) as measured by the ultraviolet absorption of microsomal lipid extracts. Figure 5 demonstrates that the appearance of enhanced quantities of the products of lipid peroxidation was quite rapid, with peak quantities detected at 30 min. The amount then decreased to control values by 12 hr. With the other three hydrocarbons, no increased amounts of lipid peroxides were detectable at any of the time intervals. The optical density values ranged from 0·151 ± 0·025 to 0·236 ± 0·053, 0·138 ± 0·016 to 0·170 ± 0·007, and 0·124 ± 0·023 to 0·208 ± 0·031 for chloroform, 1,1,2-trichloroethane and 1,1,1-trichloroethane respectively.

Figure 6 depicts the amount of diene conjugates in the microsomal lipid extracts 30 min after the various doses of carbon tetrachloride. These were significantly higher than control in the rats treated with 0·3 or 1·0 ml/kg, but not with 0·1 ml/kg.

DISCUSSION

In determining the mechanism of action of any toxic agent, temporal and dosage parameters are important considerations. These two aspects should correlate reasonably well when morphologic effects are compared to the supposed alterations in

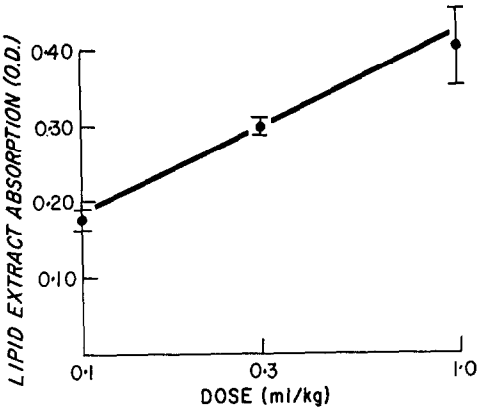

FIG. 6. Dose–response relationship for appearance of diene conjugates in microsomal lipids 30 min after administration of CCl_4. Mean ± S.E. of three rats.

biochemical function which are believed to lead to the alterations in structure. A lack of correlation can minimize cause and effect considerations, as has been demonstrated with the demise of the mitochondria as the initial site of action of carbon tetrachloride.[1, 10] The results of the present study demonstrate that triglycerides accumulate rapidly during the first 12 hr after treatment with a high dose of carbon tetrachloride. If one assumes that this accumulation is somehow associated with changes in function of the endoplasmic reticulum, one also sees that one index of its integrity, microsomal glucose 6-phosphatase activity, is also affected quite early by the treatment. Recknagel and Lombardi[11] reported significant decreases 2 and 4 hr after carbon tetrachloride, whereas we observed significant decreases from 6 to 36 hr later. Reynolds and Yee,[12] using a histochemical procedure for measuring glucose 6-phosphatase activity, detected a decrease within 1 hr after treatment.

Recknagel and Ghoshal[13, 14] have shown that hepatic microsomes prepared from rats treated with carbon tetrachloride contain more diene conjugates than normal. These products of lipid peroxidation were found to be elevated as early as 1·5 hr after treatment with carbon tetrachloride. In the present study, this observation was confirmed, and evidence of increased diene conjugation was found 15 min after treatment. Therefore, temporal changes in the endoplasmic reticulum correlate reasonably well with the reported morphologic changes observed by both light and electron microscopy[10] and are compatible with the lipoperoxidation hypothesis of Recknagel.

When one turns to the dose–response relationships, a correlation also seems to exist, although perhaps not as well as that seen with time. With glucose 6-phosphatase, we observed a significant decrease only at carbon tetrachloride doses of 0·3 ml/kg or greater. However, Reynolds and Yee,[12] using a histochemical technique, have detected decreases in the level of this enzyme after treatment with lower doses of carbon tetrachloride. When microsomal diene conjugates were measured, again we found that doses of 0·3 ml/kg or greater of carbon tetrachloride were required to show significant increases. Yet, the triglyceride data show that hepatic triglyceride accumulation occurs at doses of carbon tetrachloride below 0·3 ml/kg. In previous work,[15] it has been shown that sulfobromophthalein retention can occur in rats

after a carbon tetrachloride dose of 0·1 ml/kg. With light microscopy, morphologic changes have been observed[16] in rats treated with 0·13 ml/kg.

The correlation begins to falter when one compares the results obtained in the various parameters tested with the different halogenated hydrocarbons employed. Only carbon tetrachloride resulted in increased hepatic triglycerides, decreased microsomal glucose 6-phosphatase activity, and increased microsomal diene conjugates. Chloroform, which causes pathologic changes similar to those produced by carbon tetrachloride,[2, 3, 17] caused an increase in triglycerides, but did nothing to glucose 6-phosphatase activity or diene conjugation. Reynolds and Yee[12] also were unable to find alterations in glucose 6-phosphatase activity 1 hr after treatment with chloroform. We found no effect on either parameter 3–36 hr after chloroform treatment. Our results obtained with the 1,1,1-trichloroethane are not surprising in view of its low order of hepatotoxicity; the lesion produced by 1,1,2-trichloroethane is also milder than that produced by carbon tetrachloride.[2, 3] However, even when the hydrocarbons were added directly to liver homogenates, only those incubations containing carbon tetrachloride exhibited increased lipid peroxidation (enhanced TBA reactants). It was further shown that this effect only applied when 1–25 μl was added; with larger amounts, no increase in TBA reactants was observed. Inhibition of lipid peroxidation *in vitro* with high concentrations of carbon tetrachloride has been observed by Comparti *et al.*[7] We observed similar effects with high concentrations of chloroform and the trichloroethanes.

Thus, this study shows that, while the temporal relationships of carbon tetrachloride-induced hepatotoxicity are compatible with the lipid peroxidation hypothesis, the dosage relationships are weaker. Finally, the lack of a qualitative similarity in the results obtained with the other halogenated hydrocarbons, particularly chloroform, is disturbing. It is difficult to interpret negative data, such as the lack of effect seen with the lower doses of carbon tetrachloride and with the other hydrocarbons. Perhaps our biochemical techniques are still too insensitive to detect the absolute minimum amount of change necessary to produce a lesion, and what one measures after carbon tetrachloride is a response which is greater than that necessary to elicit the lesion. In this light, the lack of a response observed with chloroform could mean that with this agent only a minimal effect occurs, and that this effect is undetectable by our techniques, although it is sufficient to elicit the lesion. On the other hand, it might be that carbon tetrachloride and chloroform have different mechanisms of action. It has already been demonstrated in mice and dogs.[2, 3] that while the end results are qualitatively similar for both agents, quantitative comparisons of potency show that carbon tetrachloride exerts its action with doses which are about 10–13 times lower than those required for chloroform. Reynolds and Yee[12] reported that the transient influx of calcium seen after carbon tetrachloride treatment is not seen with other chloromethanes. Our present triglyceride results also indicate a difference between these agents in the time required to reach peak effect. Perhaps carbon tetrachloride has two mechanisms of action and chloroform shares only one of these mechanisms. Finally, these results could indicate that the changes seen with glucose 6-phosphatase and lipoperoxidation after carbon tetrachloride are only secondary effects and have little to do with the initiating event in the development of the lesion. Thus, it must be admitted that the molecular basis of carbon tetrachloride hepatotoxicity is not yet solved and many aspects need to be resolved.

Acknowledgement—We wish to acknowledge the excellent technical assistance of Mrs. Carol Butters and Mrs. Jana Nieman.

REFERENCES

1. R. O. Recknagel, *Pharmac. Rev.* **19**, 145 (1967).
2. C. D. Klaassen and G. L. Plaa, *Toxic. appl. Pharmac.* **9**, 139 (1966).
3. C. D. Klaassen and G. L. Plaa, *Toxic. appl. Pharmac.* **10**, 119 (1967).
4. J. L. Litchfield, Jr. and F. Wilcoxon, *J. Pharmac. exp. Ther.* **96**, 99 (1949).
5. W. M. Butler, A. M. Maling, M. G. Horning and B. B. Brodie, *J. Lipid Res.* **2**, 95 (1961).
6. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 375 (1925).
7. M. Comparti, C. Saccocci and M. U. Dianzani, *Enzymolgia* **29**, 185 (1965).
8. M. D. Comparti, R. Hartman and H. R. DiLuzio, *Lab. Invest.* **16**, 616 (1967).
9. R. G. Steele and J. G. Torrie, *Principles and Procedures of Statistics*. McGraw-Hill, New York, (1960).
10. G. L. Plaa and R. E. Larson, *Archs. envir. Hlth.* **9**, 536 (1964).
11. R. O. Recknagel and B. Lombardi, *J. biol. Chem.* **236**, 564 (1961).
12. E. S. Reynolds and A. G. Yee, *Lab. Invest.* **16**, 591 (1967).
13. R. O. Recknagel and A. K. Ghoshal, *Expl molec. Path.* **5**, 473 (1966).
14. R. O. Recknagel and A. K. Ghoshal, *Lab. Invest.* **15**, 132 (1966).
15. C. D. Klaassen and G. L. Plaa, *Toxic. appl. Pharmac.* **12**, 132 (1968).
16. R. E. Larson, G. L. Plaa and L. M. Crews, *Toxic. appl. Pharmac.* **6**, 154 (1964).
17. W. F. Von Oettingen, *The Halogenated Hydrocarbons of Industrial and Toxicological Importance*. Elsevier, Amsterdam (1964).